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(54) Title: USE OF GELATINASE B AND RELATED ENZYMES TO MOBILIZE HEMATOPOIETIC STEM CELLS FOR BLOOD STEM CELL TRANSPLANTATION

(57) Abstract

Enzymes having extracellular matrix degrading activity are used for the mobilization of hematopoietic progenitor or stem cells from the bone marrow to the blood of warm-blooded animals, in particular mammals. A matrix metalloproteinase enzyme such as gelatinase B, in particular human gelatinase B, is capable of efficiently inducing a rapid stem cell mobilization within less than an hour after a single injection. Mobilized hematopoietic progenitor and stem cells are harvested and optionally purified or enriched and then transplanted into a recipient animal, in particular human individual, for hematopoietic or bone marrow reconstitution, especially in individuals who received intensive chemo- and/or radiotherapy.

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Title: Use of gelatinase B and related enzymes to mobilize hematopoietic stem cells for blood stem cell transplantation

Field of the invention

The invention is in the field of blood stem cell and/or progenitor cell transplantation and more in particular relates to a method to rapidly mobilize (hematopoietic) stem cells from bone marrow to blood with the purpose of harvesting these cells and using them for blood stem/progenitor cell transplantation into recipients that have received lethal chemotherapy and/or radiotherapy.

Background of the invention

General introduction

The development of effective chemotherapeutic agents and radiotherapeutic regimens has resulted in a successful treatment of a variety of malignant disorders. The most successful chemotherapeutic regimens rely on combinations of agents that have different modes of action.

Over the past decades, more intensive chemotherapeutic regimens have been used that have resulted in significantly improved survival rates for a variety of malignant tumors. One of the major side effects of chemotherapy, however, is suppression of bone marrow function. In particular with more intensive regimens, patients may have reduced circulating numbers of platelets, white blood cells and red blood cells over a period of several weeks. As a consequence patients are at risk for developing bleeding and infections.

To overcome the side effects of chemotherapy, transfusions of platelets are given to prevent bleeding and patients are treated with non absorbable antibiotics to prevent systemic infections. Although sufficient for moderate dose regimens, the

more intensive chemotherapeutic regimens are associated with prolonged episodes of bone marrow depression. In such instances, the above supportive measures are insufficient to prevent complications.

Rationale of stem cell transplantation

Very intensive chemotherapy regimens or combinations of chemotherapy and radiotherapy may result in cure of the tumor. The side effects of these regimens may include permanent and complete loss of bone marrow function. To overcome the complications associated with prolonged or permanent bone marrow failure, these patients need to be transplanted with (hematopoietic) stem cells.

Transplanted stem cells are able to rapidly engraft the host, resulting in protection against the lethal complications of radiation or myeloablative chemotherapy. Another important property is their capacity of self-renewal resulting in permanent and complete repopulation of host hematopoietic tissues.

Stem cells can be harvested from the bone marrow of healthy individuals and transplanted into the recipient patient, this being called "allogeneic stem cell transplantation". Hematopoietic stem cells may also be harvested from the bone marrow of patients themselves, this being called "autologous bone marrow transplantation". Although bone marrow stem cells have the desired properties of radioprotection and long-term marrow reconstitution, hematopoietic reconstitution following these intensive treatment modalities that are lethal for the bone marrow requires a period of several weeks.

Blood stem cell transplantation

Stem cells for transplantation into the recipient patient can also be harvested from the blood. The use of such peripheral blood derived stem cells seems to achieve a more rapid restoration of marrow function in comparison with autologous

bone marrow transplantation (ABMT) (Juttner et al, 1989). Under steady state conditions the numbers of circulating hematopoietic stem cells are extremely low and insufficient to be used for transplantation purposes. Therefore, sufficient numbers of circulating stem cells can only be obtained when they are deliberately expanded. Such a "mobilization" of stem cells (release of stem cells from bone marrow to blood) can be accomplished by administering chemotherapy, hematopoietic growth factors that are involved in regulation of blood cell production (Haas et al, 1990) or both (Gianni et al, 1989). Although stem cell mobilization is a property of most hematopoietic growth factors, relatively prolonged treatment is required either without or following chemotherapy. Therefore daily monitoring of peripheral blood stem cells is required to define the optimal timing for harvesting these cells. The availability of hormones or other substances that specifically and rapidly induce mobilization in a reproducible fashion would therefore greatly facilitate the clinical application of blood stem cell transplantation.

Cytokines inducing stem cell mobilization

Previous studies in our laboratory have indicated that interleukin-1 (IL-1) may be a useful cytokine for stem cell mobilization. IL-1 is involved in host defence against infectious and inflammatory stimuli. The hematopoietic activities of IL-1 are several-fold. It induces the production of hematopoietic growth factors by a variety of accessory cells. IL-1 may also enhance the proliferative response of hematopoietic stem cells upon *in vitro* stimulation with hematopoietic growth factors. IL-1 stimulates hematopoiesis *in vivo* as well. In mice, it increases circulating levels of blood hormones, called colony-stimulating factors (CSF's), within 3 hours after a single intravenous injection. In addition, single or multiple injections of IL-1 stimulate the release of neutrophils from the

bone marrow into peripheral blood, thereby inducing neutrophilia. When administered to mice pretreated with chemotherapy (5-fluorouracil), IL-1 treatment over a period of 10 days enhances the recovery of neutrophils and other cells. The combination of IL-1 with other hematopoietic growth factors, like granulocyte colony-stimulating factor (G-CSF), acts synergistically, resulting in an even more accelerated reconstitution. We and others have shown that a single dose of IL-1 may be used to accelerate the reconstitution of neutrophilic granulocytes after neutropenia induced by the chemotherapeutic agent cyclophosphamide (Fibbe et al, 1989). Thus, IL-1 has the ability to enhance myelopoiesis in various conditions associated with neutropenia.

An increase in the number of circulating neutrophils induced by IL-1 represents one of the major hallmarks of the host defence against infections, called the acute phase response. Studies in our laboratory have indicated that IL-1 also has a similar releasing effect on the number of circulating hematopoietic (progenitor and) stem cells. Injection of a single dose of IL-1 resulted in a marked dose dependent increase in the number of neutrophils, hematopoietic progenitor and stem cells. The maximal increase was observed between 4 and 8 hours after a single injection. Transplantation of these blood derived cells into lethally irradiated recipient animals resulted in long term survival of the majority of the recipient animals (Fibbe et al, 1992). At 6 months after transplantation, the bone marrow cells of recipients were of donor origin indicating that transplantation of peripheral blood cells obtained from IL-1 treated animals resulted in long term survival of irradiated recipient animals as well as long term repopulation of their hematopoietic systems by donor derived cells.

Since an interval of 4-8 hours was observed between the injection of IL-1 and the maximum increase in the number of circulating progenitor cells, the hypothesis was presented that

an intermediate cytokine, produced in response to IL-1, may be involved. One of the cytokines considered was IL-8, a cytokine belonging to the family of chemokines.

Chemokines

Chemokines form a novel class of cytokines that have the ability to activate white blood cells, as mediators of inflammation (Van Damme, 1991; Oppenheim, 1991). Interleukin-8 (IL-8) belongs to the family of C-X-C chemokines that act mainly on neutrophils. IL-8 is rapidly produced by fibroblasts, endothelial cells, smooth muscle cells and a variety of other cells in response to other cytokines, such as IL-1 and tumor necrosis factor-α. Receptors for IL-8 are abundantly present on the surface of human neutrophils. Lower numbers of IL-8 receptors were found on various other types of leukocytes, including T lymphocytes and monocytes. Expression of receptors for IL-8 on human hematopoietic progenitor cells or stem cells has not yet been studied.

Biological activities of IL-8

In vitro IL-8 stimulates neutrophils to migrate towards the side of inflammation. In laboratory studies this effect can be measured in a so-called Boyden chamber (Van Damme et al, 1989). Neutrophils contain storaged enzymes that can be released upon stimulation with cytokines. IL-8 induces rapid degranulation and the release of certain enzymes, such as gelatinase B, β-glucuronidase, elastase, myeloperoxidase and lactoferrine. Some of these enzymes contribute to host defence functions. Other enzymes, such as gelatinase B, help neutrophils to reach the inflammatory site by degrading the matrix molecules which constitute the microenvironment of many tissues (Masure et al, 1991; Opdenakker et al, 1991).

Matrix molecules in the bone marrow microenvironment may have an important role in the regulation of hematopoiesis, since

hematopoietic stem cells are attached to these molecules. In view thereof, we hypothesized that enzymes which are involved in degrading matrix molecules might be capable of inducing the release of stem cells from the marrow to the blood.

Matrix metalloproteinases

A specific class of proteolytic enzymes are important in degrading components of extracellular matrix molecules. These enzymes are called "matrix metalloproteinases (MMP)". They play an important role in the trafficking of blood leukocytes into tissues (Matrisian et al, 1992). Various classes of MMP are commonly recognized based on the specificity of the substrate, i.e. gelatinases, collagenases and stromelysins. Several members of each class of enzymes have been purified and characterized and the genes are cloned and sequenced in humans and mice. In humans, two types of gelatinases have been purified and characterized, called the 72 kDa form (gelatinase A) and a related form with a different molecular weight of 92 kDa (gelatinase B) (Collier et al, 1988; Wilhelm et al, 1989; Huhtala et al, 1991). Most cell types, such as fibroblasts, endothelial cells and tumor cells are capable of constitutively producing gelatinase A. The production of the other 92 kDa form of the enzyme, gelatinase B, is dependent on specific stimuli. IL-8 induces within 5-10 minutes the production of gelatinase B by human neutrophils (Masure et al; Opdenakker et al, 1991). Other cytokines, like IL-1 also induce production of gelatinase B by monocytes and macrophages (Opdenakker et al, 1991b). Gelatinase activity in cell culture supernatants can be detected by SDS-PAGE zymography using gelatine as a substrate for the enzyme (Masure et al, 1990). Recently, the gene encoding the mouse gelatinase B protein has been identified and cloned from a murine cell line WEHI-3 (Masure et al, 1993). The human gelatinase B protein is not only active in human cells but also

on mouse derived cells. Thus, human gelatinase B can be used to study activities on human and primate cells.

To control the enzyme activity of these molecules, the body produces specific inhibitors of enzyme activity that are known as tissue inhibitors of metalloproteinases (TIMP). Two forms of TIMPs are recognized, called TIMP-1 and TIMP-2. Human TIMP-1 and TIMP-2 inhibit all known metalloproteinases in their activated form including both types of gelatinase. The TIMP-1 molecule specifically binds to the 92 kDa progelatinase, whereas TIMP-2 preferentially binds to the 72 kDa progelatinase. The production of these inhibitors is also tightly regulated by cytokines, such as IL-6 and IL-1 (for review, see Opdenakker and Van Damme, 1994). Recently, the mouse TIMP-1 molecule has been cloned from L929 fibroblasts (Masure et al, 1993). Therefore, recombinant protein can be used to inhibit enzyme activity of gelatinase B.

In vivo

Intravenous injection of IL-8 in animals leads to an immediate decrease in the number of circulating white blood cells (leukocytopenia). After several hours, a rebound neutrophilia is observed (Van Damme et al, 1988). The IL-8 induced neutropenia may be related to adhesion molecules that are expressed on the surface of neutrophils. IL-8 induces a rapid down-regulation and shedding of one of these molecules (L-selectin) that is involved in binding of neutrophils to the surface of blood vessels. Monkeys treated with IL-8 exhibit rapid neutrophilia with peak responses at one hour after injection and lasting 3-4 hours (Jagels and Hugli, 1992). This effect was accompanied by release of precursor cells suggesting the recruitment of cells from a reservoir in the bone marrow.

Stem cell mobilization induced by interleukin-8 in mice

Recent studies in our laboratory have shown that the chemokine IL-8 has potent stem cell mobilizing properties. At

15-30 minutes after a single intraperitoneal injection of human recombinant IL-8 at a dose of 30-100 µg/mouse, a mean 20 fold increase in the number of circulating CFU-GM was observed. This effect was specific for IL-8 and could be completely prevented by pretreating the animals with a neutralizing polyclonal anti-IL-8 antibody. Similarly, the effect was not mediated by the intraperitoneal injection itself, since saline-treated control animals exhibited no increase in the number of circulating progenitor cells. Sex-mismatched transplantation of 5×10^5 blood derived mononuclear cells obtained from male donor animals at 30 min after a single intraperitoneal injection of IL-8, into lethally irradiated (8.5 Gy) female recipients resulted in 70% survival against 22% for transplantation with an equal number of non-primed blood derived mononuclear cells ($p < 0.005$). Six months after transplantation, animals were sacrificed and chimerism was assessed using fluorescent *in situ* hybridization (FISH) with a Y-chromosome specific probe. The majority of the myeloid cells in the bone marrow, B cells in the spleen and T cells in thymus consisted of donor derived cells. These results indicate that IL-8 induces a rapid mobilization of multipotent stem cells that have radioprotective capacity and long-term marrow repopulating ability in both lymphoid and the myeloid lineages (Laterveer et al, Blood in press 1995).

Stem cell mobilization induced by interleukin-8 in primates

To study the stem cell mobilizing effect of IL-8 in primates, rhesus monkeys were injected with a single i.v. dose of 30-100 µg of IL-8 per kg body weight. IL-8 induced a profound ($0.2 \times 10^9/l$) neutropenia between 1-5 min after injection. At 15-30 min after injection of a single dose of 30 or 100 µg/kg, a reproducible 100-fold increase in the number of circulating progenitor cells to 2,800 CFU-GM/ml was observed. Neutrophilia to $30-40 \times 10^9/l$ was observed at 60-120 min after injection, lasting for 2-3 hours. These results show that IL-8 has a

similar potent and rapid stem cell mobilizing effect in primates as observed in mice and suggest that IL-8 may be potentially useful in the clinical setting of peripheral blood stem cell transplantation. Concomitant with the increase in circulating progenitor cells a dramatic increase (1,000 fold) in the circulating levels of the metalloproteinase gelatinase B was observed. Enzyme levels decreased at 2 hours after injection of IL-8, simultaneously with the decrease in the number of circulating HPC.

Brief description of the invention

The invention provides a method to rapidly mobilize hematopoietic progenitor cells and stem cells from the bone marrow to the blood in warm-blooded animals, in particular mammals such as mice, primates and men. It also comprises the harvesting and isolation of these cells from blood and the use of these cells for transplantation into recipients treated with lethal irradiation or chemotherapy.

According to the invention, enzymes that are involved in degradation of the extracellular matrix, i.e. belonging to the group of gelatinases, stromelysins, collagenases, can be used to rapidly induce mobilization of stem cells to the blood. More specifically, the invention proposes the use of the enzyme gelatinase B to induce stem cell mobilization. The invention also comprises other enzymes and other forms of these enzymes that induce rapid mobilization for a longer period of time to enable harvesting of hematopoietic progenitor cells and stem cells from the blood.

Detailed description of the invention

The subject invention provides a method for mobilizing hematopoietic progenitor or stem cells from the bone marrow to the blood in a warm-blooded animal, comprising administering to

the animal an effective amount of an extracellular matrix degrading enzyme.

The invention is not limited to particular animals and is widely applicable to any warm-blooded animal. Practically, however, the invention will be used primarily with mammals, especially human beings and certain valuable mammalian species, such as horses, dogs, cats, camels, etc. Usually, said warm-blooded animal will be a human individual.

The term "hematopoietic progenitor or stem cells" intends to cover in a broad sense all bone marrow cells which have the capability to differentiate to specialized cells, in particular to blood cells. The term covers multipotent stem cells, but also specific kinds of hematopoietic progenitor cells, including CFU-G, CFU-GM, CFU-GEMM en BFU-E.

The term "extracellular matrix degrading enzyme" intends to cover any enzym involved in the degradation of the extracellular matrix. The enzyme will usually be a matrix metalloproteinase, more particularly an enzyme selected from the group consisting of gelatinases, collagenases and stromelysins. According to the invention, said extracellular matrix degrading enzyme preferably is a gelatinase B or a modified form thereof, most preferably a human gelatinase B or a modified form thereof.

The term "modified form thereof" intends to cover all substances derived from the gelatinase B by, e.g. chemical derivation which does not substantially affect the stem cell mobilizing effect of the compound. Modifications may comprise changes in the amino acid sequence (additions, substitutions and/or deletions), the length of the molecule, the composition or structure of the carbohydrate side-chains, attachment of reactive or blocking groups or other molecules, etc.

The method of this invention preferably further comprises harvesting the mobilized hematopoietic progenitor or stem cells from the blood, and optionally purifying or enriching them.

The subject invention furthermore provides an enzyme having extracellular matrix degrading activity for use in the mobilization of hematopoietic progenitor or stem cells from the bone marrow to the blood in a warm-blooded animal, and provides the use of an enzyme having extracellular matrix degrading activity in the preparation of a pharmaceutical composition for mobilizing hematopoietic progenitor or stem cells from the bone marrow to the blood in a warm-blooded animal.

In addition the subject invention provides a pharmaceutical composition comprising an enzyme having extracellular matrix degrading activity and a pharmaceutically acceptable carrier. Such pharmaceutical composition is useful for mobilizing hematopoietic progenitor or stem cells from the bone marrow to the blood in a warm-blooded animal.

This invention also provides a pharmaceutical composition comprising hematopoietic progenitor or stem cells obtained by the above method (which includes the steps of harvesting the mobilized hematopoietic progenitor or stem cells from the blood, and optionally purifying or enriching them) and a pharmaceutically acceptable carrier. Such pharmaceutical composition may be used for effecting hematopoietic or bone marrow reconstitution.

In addition the subject invention relates to the use of hematopoietic progenitor or stem cells obtained by the above method in the preparation of a pharmaceutical composition for effecting hematopoietic or bone marrow reconstitution.

The term "pharmaceutically acceptable carrier" refers to any conventional or unconventional carrier, diluent, solvent, excipient or adjuvant appropriate for use in a pharmaceutical composition for obtaining the effects defined herein. Generally, the suitability of a carrier depends on the intended route of administration, which will usually be by injection or infusion, such as by intravenous or intraarterial injection or infusion. Suitable routes of administration and carrier materials useful with such routes of administration are well known to the person

skilled in the art and will not be detailed herein. Similarly, persons skilled in the art will easily recognize that e.g. the concentration of the enzyme resp. of the stem cells may vary between broad limits and will depend on many factors, such as the age, weight, sexe, condition, etc. of the individual to be treated with the composition. The concentration of enzyme may, e.g. vary from about 1 µg per kg body weight up to about 100 mg per kg body weight, usually from about 10 µg per kg body weight up to about 10 mg per kg body weight.

The invention circumvents the use of cytokines that are now commonly used to induce stem cell mobilization in men. This comprises the use of hematopoietic growth factors, such as human rec G-CSF, human rec GM-CSF, human rec IL-3. According to the invention, metalloproteinases, such as gelatinase B, may be used to rapidly induce stem cell mobilization within a period of from 5 to 60 minutes following a single injection. IL-8 could be used as well, but could result in many other effects, including undesirable effects.

The invention will now be explained in more detail in the following section which also contains a description of certain experiments which provide support for the claimed effect and utility of the invention. Both, the production of gelatinase B and the method to inject and use gelatinase B with the purpose of stem cell mobilization and the use of metalloproteinase-mobilized stem cells for transplantation after intensive or even principally lethal chemotherapy, radiotherapy or a combination of chemo- and radiotherapy are illustrated hereunder.

It should be understood, however, that the information given hereunder merely serves to illustrate the invention and may not be construed as limiting the invention.

Production source of gelatinase B

The experiments described herein have been carried out with gelatinase B secreted by human neutrophils. The gelatinase B was

purified to homogeneity from peripheral blood neutrophils. The purification was done by a one-step affinity chromatography on gelatin-sepharose (Masure et al., 1991). The purity was quality controlled by zymographic analysis of enzyme activity, by silver stain analysis of the electrophoretic purity of the protein and by aminoterminal sequence analysis. This gelatinase is hereafter called human neutrophil gelatinase B: it is free of human TIMP and this natural product is glycosylated by the neutrophils.

This invention is not limited, however, to the use of human gelatinase B isolated and purified from a natural source such as peripheral blood neutrophils. Instead, gelatinase B produced by recombinant DNA technology may be used, and will be used in a preferred embodiment of the invention. More particularly, it is preferred to use a gelatinase B produced by appropriately transformed eukaryotic host cells, especially mammalian cells, such as Chinese Hamster Ovary (CHO) cells or human cell cultures useful for the production of heterologous proteins. Recombinant gelatinase B produced in microorganisms such as, for example in suitable yeast cells, may likewise be useful.

Although it would be less practical, the invention includes the possibility to produce the enzyme by chemical synthesis, or by *in vitro* translation, or by whatever technique is developed for the production of proteins in general or the subject enzyme in particular.

Instead of human gelatinase B (i.e. a protein having a composition and structure which essentially corresponds to the composition and structure of human gelatinase B, such as an essentially similar amino acid composition and sequence and an essentially similar carbohydrate (or glycosylation) composition and structure), modified forms of human gelatinase B may be used, or gelatinase B enzymes derived from a different species, in particular from primates, more generally from mammals such as monkeys, pigs, cows, horses, mice, rats, goats, sheep, dogs, cats, camels, etc., including modified forms thereof.

Zymography

The activity of the described enzymes is measured by a substrate conversion assay in which gelatin is used as a substrate. Briefly, an SDS/polyacrylamide gel is copolymerised with 1 mg/ml of gelatin (warmed to 45°C). The samples to be analysed are separated in this gel by electrophoresis. Thereafter the SDS is washed out by incubation in triton X100. The enzymes are re-activated and start to degrade the copolymerized gelatin *in situ*. After overnight incubation the gelatin lysis zones can be visualized by staining of the residual substrate with Coomassie brilliant blue. The gelatinases are then visualized as clear lysis zones on a blue background and can be absolutely quantified by image analysis provided that a standard preparation of gelatinase is run on the gel. Because most body fluids (serum, synovial fluid, peritoneal fluid, cerebrospinal fluid) contain a constitutive endogenous gelatinase, called gelatinase A, which under normal conditions will not vary, this enzyme can always be used as an internal standard. Relating the (relative) activities of gelatinase B to this endogenous activity of gelatinase A circumvents errors being introduced by the sample preparation and handling.

Gelatinase inhibitors

Reversibility of the procedure claimed herein necessitates the availability of inhibitors. This will primarily be rec TIMP-1. This is prepared by expression of the mouse cDNA (Masure et al, 1993) in the methanotrophic yeast, *Pichia pastoris*. This material, hereafter called rec mouse TIMP, will be tested *in vitro* for its inhibitory activity and by staining analysis for its purity.

Pharmacokinetics of gelatinase B in the mouse

To study the pharmacokinetics of gelatinase B in the circulation, mice were injected with a bolus of human neutrophil

gelatinase B intravenously and the resulting enzyme levels were analysed by zymography. Gelatinase B was detected in the peripheral circulation.

Gelatinase activity in IL-8 injected mice

To study the gelatinase B production after injection of mice with IL-8, serum samples were drawn from IL-8 injected animals after different time intervals and analysed by zymography. Within 5 minutes after the IL-8 bolus, detectable levels of endogenous mouse gelatinase B were measured in the peripheral circulation.

Gelatinase activity in IL-8 injected monkeys

Gelatinase B activity was studied as in sub 2. after injection of rec IL-8 in monkeys. It was observed that a rapid and transient increase of gelatinase B activity remained detectable for about 4 hours and peaked to levels over a thousand fold over base level concentrations within minutes after the IL-8 bolus.

Simultaneous induction of circulating gelatinase B and stem cell mobilization in Rhesus monkeys

Recombinant human IL-8 was administered as a single intravenous injection at doses of 10, 30 and 100 mg/kg body weight to Rhesus monkeys (age 2-3 years, body weight 2.5-4.5 kg). Venous blood samples were obtained at time intervals ranging from 1 to 480 minutes after injection of IL-8. Peripheral blood cell counting and colony assays, as well as immunotyping were performed. Hematopoietic progenitor cells were assessed using a semisolid culture assay in methylcellulose. Cells were cultured in 3.5 cm dishes, in 1 ml medium containing 10 ng/rhuGM-CSF, 10 ng/rhuG-CSF, 10 ng/rhuSCF, 2 U/rhEPO and 15 ng rhuIL-3, 10^{-5}M β-mercaptoethanol, 500 ng transferrin, 1.1% methylcellulose and 20% V/V human plasma in IMDM. Blood cell samples were plated in triplet at a concentration of $5x10^5$ cells/ml. After 7-8 days of culture

at 37°C, 5% CO_2 in a fully humidified atmosphere, the numbers of colony-forming units were scored using an inverted microscope.

IL-8 injection resulted in a dose dependent increase in the numbers of circulating progenitor cells starting at 5 minutes after injection and reaching maximum levels at 30 minutes after injection. A mean number of 1,382 ± 599 colony forming units per ml blood was reached after an injection of 100 µg IL-8/kg body weight (mean ± SD of 8 experiments). In control animals injected with vehicle alone the mean number of colony forming units per ml blood was 9 ± 7. The increment in the absolute numbers of progenitor cells ranged from 10 to 100-fold at a dose of 100 µg/kg IL-8. Colony assays revealed that all types of hemato-poietic progenitor cells including CFU-G, CFU-GM, CFU-GEMM and BFU-E were mobilized. IL-8 did not result in a shift of the type of colony forming cells.

Monkeys were sequentially injected with multiple doses of IL-8. In these experiments time intervals between the first and second injection were varied. Irrespective of the time interval between the first and second injection of IL-8, neutrophilia was observed. The increment in the number of circulating stem cells however varied depending on the time interval. Monkeys given two injections of IL-8 with an interval of 4 hours had a significant increase in the number of circulating progenitors after the first injection, but no increase after the second injection. Animals given a second injection of IL-8 at 24 hours after the first injection of IL-8 had an intermediate increase in progenitor cells after the second injection. When the intervals were prolonged to 72 hours the numbers of progenitor cells were equally increased after the first and second injection. A similar response pattern was observed for the circulating levels of gelatinase B. With a time interval of 4 hours between the first and second injection of IL-8 almost no increase in gelatinase B was observed. At 24 hours an intermediate increase was observed while after an interval of 72 hours the gelatinase

B increase was identical after both injections. These data are compatible with the hypothesis that induction of gelatinase B was responsible for the stem cell mobilizing effect of IL-8.

Repopulating ability of IL-8 mobilized stem cells in mice

IL-8 was administered at doses ranging from 0.1 to 100 µg i.p. per mouse to male Balb-C mice (age 8-12 weeks, weight 12 to 25 g). Animals were sacrificed at time intervals ranging from 1 to 240 minutes after IL-8 administration and blood, bone marrow and spleen cells were harvested. Transplantation of 1.5×10^6 mononuclear cells obtained from male donor animals at 30 minutes after a single i.p. injection of 30 µg of IL-8, into lethally irradiated female recipient animals resulted in 100% survival (n=10). In control animals transplanted with an equal number of mononuclear cells derived from saline-treated donor animals, no survival was observed. At 6 months after transplantation female recipients of mononuclear cells derived from IL-8 treated male donors were sacrificed and chimerism was determined in bone marrow, spleen and thymus. Bone marrow cells (83 ± 25%), spleen cells (89 ± 5%) and thymus cells (64 ± 28%) mainly consisted of Y-chromosome positive cells showing that the IL-8 mobilized cells not only had radioprotective capacity but also long-term myelolymphoid repopulating ability.

Stem cell mobilization by administration of gelatinase B

Human neutrophil gelatinase B was dissolved in phosphate-buffered saline (PBS) at a concentration of 1 mg/ml. This stock solution was further diluted in PBS to a total volume of 10 ml. Monkeys were then injected intravenously over a time course of 30-60 seconds with gelatinase B at a dose of 10-100 µg/kg body weight. At various time intervals after intravenous injection, blood samples were drawn to assess the numbers of progenitor cells.

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Claims

1. A method for mobilizing hematopoietic progenitor or stem cells from the bone marrow to the blood in a warm-blooded animal, comprising administering to the animal an effective amount of an extracellular matrix degrading enzyme.

2. The method of claim 1 wherein said warm-blooded animal is a mammal.

3. The method of claim 1 wherein said warm-blooded animal is a human individual.

4. The method of claim 1 wherein said extracellular matrix degrading enzyme is a matrix metalloproteinase.

5. The method of claim 1 wherein said extracellular matrix degrading enzyme is selected from the group consisting of gelatinases, collagenases and stromelysins.

6. The method of claim 1 wherein said extracellular matrix degrading enzyme is a gelatinase B or a modified form thereof.

7. The method of claim 1 wherein said extracellular matrix degrading enzyme is a human gelatinase B or a modified form thereof.

8. The method of claim 1 further comprising harvesting the mobilized hematopoietic progenitor or stem cells from the blood.

9. The method of claim 1 further comprising harvesting the mobilized hematopoietic progenitor or stem cells from the blood and purifying or enriching them.

10. An enzyme having extracellular matrix degrading activity for use in the mobilization of hematopoietic progenitor or stem cells from the bone marrow to the blood in a warm-blooded animal, preferably a mammal, more preferably a human individual.

11. The enzyme of claim 10 which is a gelatinase B or modified form thereof, preferably a human gelatinase B or modified form thereof.

12. Use of an enzyme having extracellular matrix degrading activity in the preparation of a pharmaceutical composition for mobilizing hematopoietic progenitor or stem cells from the bone marrow to the blood in a warm-blooded animal, preferably a mammal, more preferably a human individual.

13. The use of claim 12 wherein said enzyme is a gelatinase B or modified form thereof, preferably a human gelatinase B or modified form thereof.

14. A pharmaceutical composition comprising an enzyme having extracellular matrix degrading activity and a pharmaceutically acceptable carrier.

15. The pharmaceutical composition of claim 14 wherein said enzyme is a gelatinase B or modified form thereof, preferably a human gelatinase B or modified form thereof.

16. The pharmaceutical composition of claim 14 for mobilizing hematopoietic progenitor or stem cells from the bone marrow to the blood in a warm-blooded animal, preferably a mammal, more preferably a human individual.

17. A pharmaceutical composition comprising hematopoietic progenitor or stem cells obtained by the method of claim 8 or claim 9 and a pharmaceutically acceptable carrier.

18. The pharmaceutical composition of claim 17 for effecting hematopoietic or bone marrow reconstitution.

19. Use of hematopoietic progenitor or stem cells obtained by the method of claim 8 or claim 9 in the preparation of a pharmaceutical composition for effecting hematopoietic or bone marrow reconstitution.

INTERNATIONAL SEARCH REPORT

Int' onal Application No
PCT/NL 96/00149

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/48 A61K35/28 A61K35/14

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BLOOD, vol. 79, no. 3, 1 February 1992, NEW YORK, N.Y., US, pages 657-665, XP002006639 M. KOENIGSMANN ET AL.: "MYELOID AND ERYTHROID PROGENITOR CELLS FROM NORMAL BONE MARROW ADHERE TO COLLAGEN TYPE I." see page 660, left-hand column, line 16 - right-hand column, line 15 see page 663, right-hand column, paragraph 4 - page 664, left-hand column, paragraph 1 --- -/--	1-9

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
26 June 1996	18.07.96
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Ryckebosch, A

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 198, no. 2, June 1991, BERLIN, DE, pages 391-398, XP002006640 S. MASURE ET AL.: "PURIFICATION AND IDENTIFICATION OF 91-kDa NEUTROPHIL GELATINASE: RELEASE BY THE ACTIVATING PEPTIDE INTERLEUKIN-8." cited in the application see the whole document ---	1-13
A	JOURNAL OF IMMUNOLOGY, vol. 148, no. 2, 15 January 1992, BALTIMORE US, pages 417-421, XP002006641 W.E. FIBBE ET AL.: "SUSTAINED ENGRAFTMENT OF MICE TRANSPLANTED WITH IL-1-PRIMED BLOOD-DERIVED STEM CELLS." cited in the application see the whole document ---	17-19
X	DE,A,19 24 715 (WORTHINGTON BIOCHEMICAL CORP.) 27 November 1969 see claim 1 ---	14
P,X	BLOOD, vol. 85, no. 8, 15 April 1995, NEW YORK, N.Y., US, pages 2269-2275, XP002006642 L. LATERVEER ET AL.: "INTERLEUKIN-8 INDUCES RAPID MOBILIZATION OF HEMATOPOIETIC STEM CELLS WITH RADIOPROTECTIVE CAPACITY AND LONG-TERM MYELOLYMPHOID REPOPULATING ABILITY." see page 2273, line 5 - line 19 -----	1-19

1

INTERNATIONAL SEARCH REPORT

national application No.

PCT/NL96/00149

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 1-9
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 1-9 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

Int onal Application No
PCT/NL 96/00149

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
DE-A-1924715	27-11-69	CA-A-	1014068	19-07-77
		FR-A-	2008611	23-01-70
		GB-A-	1251398	27-10-71